Stenotrophomonas maltophilia plays an important role as an opportunistic pathogen in immunocompromised individuals. Despite its clinical implications, information regarding its pathogenicity remains unclear. Various methods have been employed to demonstrate that this bacterium is pathogenic. However, the debate as to whether S. maltophilia is a true pathogen or a colonizer still continues, as effective killing was not seen in earlier experiments with different animal models of infection (Denton and Kerr, 1998; Adamak et al., 2011; Pomplio et al., 2011). A study using a murine lung infection model illustrated that different strains of mice exhibited different outcomes after S. maltophilia infection (Rouf et al., 2011). Strains such as A/j and DBA/2 were permissive for clinical isolates of S. maltophilia and showed that higher levels of proinflammatory cytokines such as IL-6, TNF-α and IL-1β were found to be elevated. In contrast, BALB/c and C57BL/6 strains were non-permissive for S. maltophilia. Nematotoxic activity by an environmental S. maltophilia strain has been shown against a free-living nematode, Panagrellus redivivus, and against a plant-parasitic nematode, Bursaphelenchus xylophilus (Huang et al., 2009).

Caenorhabditis elegans is well known as a model of infection for bacterial pathogenesis (Zak & Sande, 1999). The mode of pathogenesis in C. elegans could be associated with infection in the intestine or cuticle; it may be toxin mediated or may be due to multiple or unknown methods of killing (Darby, 2005). Many bacteria and fungi have been studied extensively to evaluate the mode of killing in C. elegans (Powell & Ausubel, 2008). The use of both a toxin-mediated pathway and slow killing by colonization of C. elegans have been observed for Pseudomonas aeruginosa (Tan et al., 1999). Other bacteria, like Salmonella species, have been reported to colonize the intestine of C. elegans (Aballay et al., 2000; Labrousse et al., 2000), whilst Yersinia species colonize the cuticle of C. elegans, especially the mouth, by producing biofilm (Darby et al., 2002; Joshua et al., 2003). Burkholderia species tend to kill C. elegans either by infection or intoxication, or even by both methods (Darby, 2005).

Sixty-nine isolates of S. maltophilia obtained from various clinical sources such as tracheal aspirate, urine and pus were investigated. All isolates previously identified to the species level by phenotypic methods were reconfirmed genotypically as S. maltophilia by species-specific PCR using primers (SM1 5’-CAGCCTGCGAAAGTA-3’ and 5’-SM4 TTAAGCTTGCCAGAAGAG-3’) as described by Whitby et al. (2000). These clinical strains were further classified as invasive or non-invasive based on the anatomical site of infection. Invasive strains included in the study were those that were isolated from sterile sites such as peripheral blood and cerebrospinal fluid. Non-invasive strains were those that were isolated from non-sterile sites such as tracheal aspirate, wound swabs, and pus. A single strain of P. aeruginosa ATCC 27853 was used as a positive control, whilst a single strain of Escherichia coli OP50 was used as a negative control and as a food source (Brenner, 1974) for the nematode killing assay. E. coli OP50 is a uracil-dependent mutant of E. coli, bacterial overgrowth on the nematode growth medium (NGM) is thus restricted as NGM has limited uracil. All strains (S. maltophilia ATCC 13637, P. aeruginosa ATCC 27853 and E. coli OP50) maintained in 20% glycerol in Luria–Bertani (LB) broth (Difco Laboratories) were grown statically overnight from a single colony in 2 ml LB broth at 37 °C.

The nematode C. elegans N2 Bristol strain and E. coli OP50 (kindly provided by Leo Eberl, University of Zürich, Zürich, Switzerland) were maintained under standard culture conditions on NGM agar with E. coli OP50 as a food source (Sulston & Hodgkin, 1988). Worms were synchronized by performing a timed egg-laying experiment using fluorodeoxyuridine treatment of gravid adults, hatching of the eggs overnight in M9 minimal medium and plating first larval stage worms onto lawns of E. coli on NGM plates (Sutphin & Kaeberlein, 2009). Synchronized worms were grown to the fourth larval stage or young adult stage at 25 °C for use in the killing assays.

The in vivo killing efficiency of S. maltophilia was evaluated by four different methods: a classical fast killing assay, a fast killing assay using a nitrocellulose filter, a slow killing assay and a virulence assay using heat-killed strains. The classical fast killing assay was conducted according to the method of Mahajan-Miklos et al. (1999) where 50 μl of the bacterial test culture grown overnight in LB broth were spread on NGM plates containing 0.15 M sorbitol. For the killing assay using a nitrocellulose filter, the filter paper was placed on the agar plate and the test bacteria were spread evenly onto the nitrocellulose filter (pore diameter 0.45 μm). In the slow killing assay, test bacteria were spread on normal NGM plates (without sorbitol) and incubated at 37 °C for 24 h. For the killing assay using heat-killed strains, a single colony of S. maltophilia strain was grown in 5 ml Mueller–Hinton broth overnight. The bacterial cells were killed by boiling for 20 min followed by centrifugation for 15 min at 900 x g. The complete death of the bacteria was ensured when no colonies appeared on Mueller–Hinton agar streaked with a loop of boiled bacterial cells. NGM plates were inoculated with a drop of the heat-killed cell pellet resuspended at ten times the original density. The plates were incubated at 37 °C for 24 h and then placed at room temperature for 8–12 h (Kurz et al., 2003).

For all four methods, after incubating the plates at 37 °C for 24 h to ensure the
growth of the test organism, 30 synchronized fourth larval stage worms were picked and placed on the test bacteria using a loop (Mahajan-Miklos et al., 1999). A worm was considered alive when it responded to touch but was scored dead when no movement was seen upon external stimulation. The nematocidal activity of different invasive and non-invasive strains of *S. maltophilia* was scored at different time points (0, 6, 12, 24, 48 and 72 h) and the data were recorded. Worms that stuck to the walls of the plate and died were excluded from the study.

The results are illustrated in Fig. 1. Of the invasive isolates, 71.6% were found to be lethal by the classical fast killing method at 72 h. The slow killing method showed a death rate of only 28% at 72 h for both invasive and non-invasive strains. However, complete killing was seen after longer time points (10–14 days). The filter method was seen to be efficient where lethality was observed at 24 h. Approximately 75.2% of nematodes were killed by the invasive strains and 72% by the non-invasive strains at 24 h for the filter method. This could be attributed to the fact that the culture supernatant of *S. maltophilia* exhibits haemolytic and enzymatic activities that cause vigorous endocytosis and cell aggregation as seen in HeLa and Vero cells (Figueirêdo et al., 2006). The concentrated enzymes would have passed through the filter paper causing effective killing of *C. elegans*. This also demonstrated that direct bacterial contact is not required for the nematodes to die (Mahajan-Miklos et al., 1999).

For the heat-killed method at the 24 h time point, most of the nematodes were killed by invasive (75.5%) and non-invasive (72.2%) strains. On further incubation, the invasive strains showed 80 and 87% lethality at 48 and 72 h, respectively, whilst non-invasive isolates showed 76.2 and 84.2% lethality at 48 and 72 h, respectively. In the case of the filter method, 75.2 and 72% of nematodes were killed by invasive and non-invasive strains, respectively, at the 24 h time point. Nevertheless, at longer time points (48 and 72 h), invasive strains showed 80.2 and 87% lethality in the nematodes using the filter method, and non-invasive strains showed 77 and 84.2% lethality.

The kinetics of *C. elegans* killing are shown in Fig. 1. The invasive strains effectively killed the nematodes in the filter-based method and heat-killed method in comparison with the other two methods tested. The killing assays were done

![Fig. 1. Different methods employed in *C. elegans* killing. *C. elegans* killing assay using: (a) the fast killing method, (b) slow killing method, (c) heat-killed method and (d) filter-based method. Vertical bar represents SD. Experiments were conducted in triplicate. *, *E. coli* OP50 strain; ■, *S. maltophilia* ATCC 13637; ▲, *P. aeruginosa* ATCC 27853; ●, invasive strains; ●, non-invasive strains. The invasive and non-invasive classification is based on the anatomical site of isolation for the different clinical isolates of *S. maltophilia*.](image)
according to Tan et al. (1999), except that we used P. aeruginosa ATCC 27853 instead of P. aeruginosa PA14. The results obtained in this study were in accordance with those reported by Tan et al. (1999) and Fouhy et al. (2007). However, for the S. maltophilia strains used in this study, further incubation was required to obtain complete killing.

In conclusion, the filter method and heat-killed method showed similar results where complete killing was observed at 24 h. It is recommended to use a filter-based method or heat-killed method for routine screening of S. maltophilia for pathogenesis. The cellular components that were heat stable and extracellular proteins that could pass through a filter of pore diameter 0.45 μm need to be studied to understand the virulence properties of S. maltophilia.

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